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# Highly sensitive high-performance liquid chromatographic assay for methotrexate in the presence and absence of anti-methotrexate antibody fragments in rat and mouse plasma

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**Abstract**

Recently, Balthasar and Fung have proposed that anti-methotrexate antibody fragments may be employed to enhance the selectivity of intraperitoneal methotrexate (MTX) therapy. This current work presents a sensitive high-performance liquid chromatographic method for measuring plasma concentrations of total (i.e., bound and unbound) MTX and free (unbound) MTX in rat and mouse plasma, in the presence or absence of therapeutic anti-MTX antibody fragments. The assay involves pre-column derivatization of MTX by sodium hydrosulfite to 2,4-diamino-6-methylpteridine. The limit of quantitation for MTX by this assay was 1.25 ng in rat plasma, mouse plasma and mouse plasma ultrafiltrate, which corresponds to a concentration of 25 ng/ml for a 50 μl sample. The limit of quantitation was found to be 2.5 ng in rat plasma ultrafiltrate (i.e., 50 ng/ml in 50 μl rat plasma ultrafiltrate). The method was shown to be quite accurate, as the mean assayed concentration of quality control samples was within 10% of theoretical values. We have applied the method to the investigation of MTX pharmacokinetics in mice and rats, following the administration of MTX alone or following simultaneous administration of MTX and anti-MTX Fab fragments. The method has been shown to be suitable for the assay of total and free methotrexate in the plasma of these species and will enable the testing of pharmacokinetic hypotheses regarding the influence of anti-MTX Fab fragments on the disposition of MTX. 

*Keywords:* Methotrexate; Anti-methotrexate antibody fragments

## 1. Introduction

Traditional drug targeting approaches attempt to enhance the selectivity of drug action by increasing the efficiency of drug delivery to desired sites of drug activity. Conversely, inverse targeting strategies attempt to increase selectivity by reducing the efficiency of drug delivery to sites associated with drug toxicities. Recently, Balthasar and Fung have demonstrated the utility of anti-methotrexate (anti-MTX) antibodies and antibody fragments in an inverse targeting strategy designed to enhance the pharmacokinetic selectivity of intraperitoneal MTX therapy for peritoneal tumors [1]. Their inverse targeting approach calls for simultaneous intravenous administration of anti-MTX antibodies and intraperitoneal administration of MTX. The presence of anti-MTX antibodies in systemic circulation leads to a rapid complexation of MTX diffusing out of the peritoneum and entering the blood. However, the large molecular mass of the anti-MTX antibodies

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**EXHIBIT 1**

limits their rate of entry into the peritoneal cavity; consequently, this strategy produces regiospecific alterations in MTX binding. This selective alteration of drug binding has been shown to produce dramatic, regiospecific alterations in the pharmacokinetics of the target drug. Specifically, it has been shown that systemic administration of anti-MTX antibodies may decrease maximal free MTX plasma concentrations and also decrease the cumulative systemic exposure to free MTX, while not altering peritoneal exposure to free MTX. Consequently, this combined therapy has been shown to increase the pharmacokinetic selectivity of intraperitoneal chemotherapy by decreasing the fraction of drug available for distribution to sites associated with systemic toxicities.

These preliminary studies have suggested that therapeutic benefits may result from the proposed inverse targeting strategy; however, many questions remain regarding proposed pharmacokinetic and therapeutic hypotheses. Testing proposed pharmacokinetic hypotheses requires the availability of a sensitive assay capable of accurately quantifying both total and unbound concentrations of MTX in plasma. Preliminary studies had been conducted with the use of radio-labeled MTX [1]; however, administration of radio-labeled drug to animals is inconvenient, expensive and raises safety concerns. Consequently, we had hoped to identify a suitable MTX assay for use in our pharmacokinetic investigations.

Numerous methods for MTX analysis have been reported in the literature. These include high-performance liquid chromatography (HPLC) analysis with ultraviolet detection [2–6] and fluorescence detection [7–10], radioimmunoassay [11–14], dihydrofolate reductase inhibition assay [15], enzyme-multiplied immunoassay [16], fluorescence polarization immunoassay [17], enzyme immunoassay [18], and capillary zone electrophoresis with laser-induced fluorescence detection [19]. For our purposes, HPLC approaches provide the greatest compromise between sensitivity, selectivity, assay time and assay expense.

The most sensitive HPLC assays have employed either pre-column [7,8] or post-column derivatization [20–23] of MTX coupled with fluorescence detection. However, no HPLC assay has been validated for application to the quantitation of ng/ml concentrations of MTX in rat and mouse plasma. Moreover, no HPLC assay has been shown to be capable of accurately measuring total and unbound MTX concentrations in the presence of therapeutic anti-MTX antibodies. Consequently, we have attempted to modify existing assays for these applications.

In our search to find a sensitive HPLC assay that could detect MTX both in plasma and plasma ultrafiltrate, we found that the assay reported by Deen et al. appeared most useful [8]. This HPLC assay involved pre-column derivatization of MTX, followed by fluorescence detection. The method reported a limit of detection of 2.3 ng/ml for MTX starting with 1.0 ml of human plasma. Although this method was not as sensitive as some of the post-column derivatization assays reported [21], it was simple and could be implemented with standard HPLC equipment. Unfortunately, when we applied the method to the analysis of MTX in rat plasma we observed lower sensitivity compared to results reported by Deen et al. [8], inconsistent recoveries and high variability. We have, however, successfully adapted the method of Deen et al. for our purposes.

In this paper, we describe a simple and convenient HPLC procedure that allows the determination of MTX in the presence and absence of anti-MTX antibody. The method utilizes pre-column derivatization of MTX by sodium hydrosulfite to a fluorescent derivative 2,4-diamino-6-methylpteridine as reported previously [8]. The assay was validated for its reproducibility and accuracy over a concentration range of 25–500 ng/ml. The limit of quantitation for MTX by this assay was 1.25 ng in rat plasma, mouse plasma and mouse plasma ultrafiltrate, and 2.5 ng in rat plasma ultrafiltrate.

## 2. Experimental

### 2.1. Chemicals and reagents

Methotrexate (>98% pure, HPLC), folic acid (approximately 98% pure), and sodium hydrosulfite were purchased from Sigma (St. Louis, MO, USA). Methanol was HPLC grade and all other chemicals were analytical grade. Anti-MTX Fab fragments (AMF) were produced in our laboratory and characterized as previously described [24].

### 2.2. Chromatographic instrumentation and system

The HPLC system consisted of Waters (Milford, MA, USA) components, including a Novopak $C_{18}$ column (150×3.9 mm), a Model 515 pump, a Model 474 scanning fluorescence detector and a Model 717 autosampler. Waters Millenium32 software was used for instrument control, data acquisition and processing.

The mobile phase comprised of 0.1 *M* Tris (trishydroxymethylaminomethane) buffer (pH 7.0)–methanol (9:1), and was filtered (0.45 μm pore size) and degassed prior to use. A constant mobile phase flow-rate of 1 ml/min was used for separation. The fluorescence detector was set at excitation and emission wavelengths of 367 nm and 463 nm, respectively.

### 2.3. Sample preparation

#### 2.3.1. Standards and quality control samples

Stock solutions of MTX (50 μg/ml) and folic acid (50 μg/ml) were prepared in distilled water and stored at 4°C. Working standards were prepared in the concentration range of 15.6–500 ng/ml with phosphate-buffered saline (PBS, pH 7.4). Folic acid at 1.0 or 5.0 μg/ml was used as an internal standard. Quality control samples (QCs) for MTX were prepared by spiking rat/mouse plasma with appropriate amounts of MTX. QCs for AMF containing MTX were prepared by spiking plasma containing 20% AMF with MTX. Filtered rat/mouse plasma was obtained by ultrafiltration in Centrifree tubes (30 000 molecular mass cutoff, YM membrane; Amicon, Beverly, MA, USA) at 25°C at 1000 *g* for 30 min.

#### 2.3.2. Derivatization

(A) For total MTX in rat plasma, reduction of MTX was carried out in glass vials. A reaction solution was prepared containing 100 μl plasma (blank rat plasma in case of standards), 25 μl of folic acid (1.0 μg/ml), 100 μl of PBS (containing MTX in case of standards), 100 μl of 2 *M* sodium acetate–5 *M* acetic acid buffer (pH 6.0), 50 μl of freshly prepared sodium hydrosulfite (10 mg/ml) solution. The solution was heated at 92°C for 20 min. The solution was then cooled to room temperature, centrifuged at 200 *g* for 2 min. A 175-μl aliquot of the supernatant was injected on the column.

(B) For total MTX in mouse plasma, reduction of MTX was carried out as above with slight modification. A solution was prepared to contain 50 μl plasma (blank mouse plasma in case of standards), 25 μl of folic acid (5.0 μg/ml), 100 μl of PBS (containing MTX in case of standards), 75 μl of 2 *M* sodium acetate–5 *M* acetic acid buffer (pH 6.0) and 50 μl of freshly prepared sodium hydrosulfite solution (10 mg/ml). The solution was heated at 92°C for 30 min. The solution was cooled, centrifuged, and 200 μl of the supernatant was injected on the column.

(C) For free MTX in rat/mouse plasma, starting with 50 μl of ultrafiltrate rat/mouse plasma, the procedure for sample preparation was the same as described in for total MTX in mouse plasma.

### 2.4. Assay validation

Assay validation was performed by assessing the intra- and inter-day accuracy and precision in quantifying MTX in QCs. Intra-day variability was assessed through the analysis of QCs in triplicate, and inter-day variability was determined through the analyses of QCs on three different days. Accuracy was determined by calculating the concentration present using standard curve and comparing it to known (spiked) concentrations. The limit of quantitation was defined as the MTX concentration in the lowest QC yielding an intra-assay relative standard deviation (RSD) less than 10% and producing a mean assayed concentration within 10% of the theoretical concentration.

### 2.5. MTX pharmacokinetics in mice

An i.p. bolus of 10 mg/kg MTX in sterile saline solution was administered to a group of three male Swiss–Webster mice. Blood samples (20–60 μl) were obtained from the saphenous vein at 15, 30, 60, 120, 180, 240 min. Plasma was separated from blood samples via centrifugation at approximately 10 000 *g* for 2 min. Samples were diluted, if appropriate, and analyzed for MTX concentration.

### 2.6. MTX pharmacokinetics in absence and presence of anti-MTX Fab in the rat

An abdominal aortal cannula was inserted into two female Sprague–Dawley rats (225–250 g) under ketamine (50 mg/kg, Fort Dodge Labs., Fort Dodge, IA, USA) and xylazine (10 mg/kg, Miles, Shawne Mission, KA, USA) anesthesia. MTX, 2.0 μmol/kg (909 μg/kg) in sterile saline, was administered as an intraarterial (i.a.) bolus, followed with continuous i.a. infusion of sterile saline over 6 h. Blood samples (0.1–0.3 ml) were drawn from the aortal cannula at 15, 30, 60, 90, 120 min and placed in heparinized microcentrifuge tubes. After centrifugation, aliquots of plasma were analyzed for total MTX concentration. Analysis of MTX in the presence of AMF was demonstrated by administering continuous i.a. infusion of 0.4 μmol/kg AMF for 6 h with an i.a. bolus of MTX (2.0 μmol/kg) at 4 h of the infusion. Free MTX concentration was determined following ultrafiltration of 100 μl of plasma at 1000 *g* for 30 min in Centrifree tubes at 25°C.

## 3. Results and discussion

### 3.1. Development of the assay

Deen et al. introduced a sensitive HPLC method for the analysis of MTX in human plasma which included a pre-column reduction of MTX to a fluorescent product [8]. However, when we applied this procedure to small volumes of rat and mouse plasma, we observed high variability and lower sensitivity than that reported by Deen et al. The authors used 70% perchloric acid to deproteinate the plasma samples, and then added NaOAc–NaOH buffer to neutralize samples to pH 5.6–6.0 prior to derivatization. Also, Deen et al. reported that the derivatization reaction was highly pH dependent, with a reaction maximum near pH 6.0 [8]. Preliminary studies in PBS confirmed this observation (data not shown). However, we found that we could reduce assay variability by eliminating the acidification and neutralization steps. In rat plasma, maximum MTX response was achieved near pH 5.0 as shown in Fig. 1. In addition, we found that we could

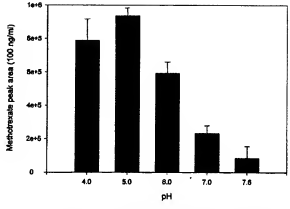


Fig. 1. The effect of pH on the derivatization reaction was investigated by assessing detector response from rat plasma samples containing MTX (100 ng/ml). Reaction solutions were buffered to pH 4, 5, 6, 7, or 7.6 ($n$=3).

reduce assay variability by lowering the reaction temperature from 100°C to 92°C and increasing the reaction time from 15 to 30 min (Fig. 2). The original method used a mobile phase of 0.005 *M* tetrabutylammonium phosphate in water with 25% methanol at 1.4 ml/min; however, we found that we could reduce the assay run-time while maintaining resolution by using a methanol–Tris buffer (pH 7.0)

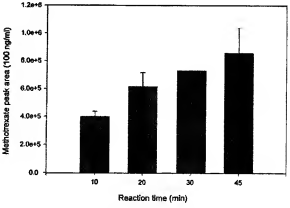


Fig. 2. The effect of reaction time on the completeness and variability of the derivatization reaction was investigated by assessing detector response from rat plasma samples containing MTX (100 ng/ml). The derivatization reaction was allowed to proceed for 10, 20, 30 or 45 min ($n$=3). The 30 min reaction time provided the most desirable combination of response magnitude and variability for our purposes.

mobile phase at a flow-rate of 1 ml/min. This modification reduced the run-time from approximately 15 min (as reported by Deen et al.) to approximately 8 min.

It was interesting to find that the authors reported higher fluorescence response (four times) in PBS as compared to plasma; in our hands, we observed a greater slope in the linear relationship between detector response and MTX concentration in plasma samples relative to PBS samples (plasma slope= 0.0085, $r^2$=0.992; PBS slope=0.0052, $r^2$=0.948). We then proceeded to investigate the effect of protein and plasma on the fluorescence response from MTX samples. The slope of the standard curves increased in the order of bovine serum albumin (3.1%)>bovine serum albumin (6.7%)>rat plasma. Assay response was shown to be dependent on the inclusion of plasma and on the concentration of bovine serum albumin added to the reaction mixture. Therefore, we decided to use standards containing rat plasma to analyze MTX in rat plasma and standards containing mouse plasma to analyze MTX in mouse plasma. Standards containing plasma ultrafiltrate produced poor correlations between concentration and response. However, we found that we could stabilize the reaction and improve assay response through the addition of rat or mouse plasma to the reaction mixture. Fig. 3 shows typical chromatograms obtained from HPLC analysis of mouse and rat plasma containing MTX. Retention times were approximately 3 and 7 min for folic acid (the internal standard) and MTX, respectively. There was no interference from either reaction products or endogenous substances.

Although the majority of methotrexate is eliminated unchanged in man and in rodents, pharmacokinetic investigations conducted in these species have demonstrated that a fraction of MTX is metabolized to 7-hydroxymethotrexate and 2,4-diamino-$N^{10}$-methylpteroic acid. For example, following high-dose MTX therapy in man, approximately 70–94% of the dose is recovered as the parent compound, 0.4–11% as 7-hydroxymethotrexate, and 0.01% as 2,4-diamino-$N^{10}$-methylpteroic acid [25]. Following MTX doses of 4–10 mg/kg in rats, about 80–90% of the dose was eliminated as the parent compound and approximately 10% was metabolized to 7-hydroxymethotrexate [26,27]. Unfortunately, 7-hydroxymethotrexate is not commercially available and, consequently, we were unable to assess assay selectivity in samples containing both MTX and 7-hydroxymethotrexate. However, Bremnes et al. have found that the concentrations of the major MTX metabolite, 7-hydroxymethotrexate, are only 1–10% of the concentrations of parent drug following a 10 mg/kg i.v. bolus dose in rats [26]. Consequently, in spite of our inability to assure assay selectivity, we do not expect significant interference from methotrexate metabolites in our proposed preclinical investigations.

### 3.2. Validation of the assay

Calibration curves were linear over the concentration range of 15.6 to 500 ng/ml in mouse and rat plasma (typically, $r^2$ values were greater than 0.995). The intra-day and inter-day RSDs for total MTX in rat plasma were less than 8%, and less than 9% for mouse plasma (Tables 1 and 2). For free MTX in rat and mouse plasma ultrafiltrate, RSDs were less than 11% (Table 3). The recoveries of free and total MTX from spiked rat and mouse plasma were within 10% of theoretical values. The limit of quantitation for MTX by this assay was 1.25 ng in rat plasma, mouse plasma, and mouse plasma ultrafiltrate, which corresponds to a concentration of 25 ng/ml for a 50 μl sample. The limit of quantitation was found to be 2.5 ng in rat plasma ultrafiltrate (i.e., 50 ng/ml in 50 μl rat plasma ultrafiltrate).

### 3.3. Pharmacokinetics in mice

The MTX concentration time profile following an i.p. bolus of 10 mg/kg MTX in Swiss–Webster mice is shown in Fig. 4. The data was fitted to a two compartment model using the Scientist program (Micromath, Salt Lake City, UT, USA). The fitted parameters were clearance (2.22±0.22 l/kg/h) and volume of distribution (0.80±0.53 l/kg). The half life of MTX in these mice was found to be 36.8±7.6 min. These values are very similar to those reported by Osman et al. for an i.p. bolus of 50 mg/kg in female Swiss albino mice with clearance at 1.85±0.07 l/kg/h, volume of distribution of 1.28±0.14 l/kg and half life of 28.4±2.4 min [28].

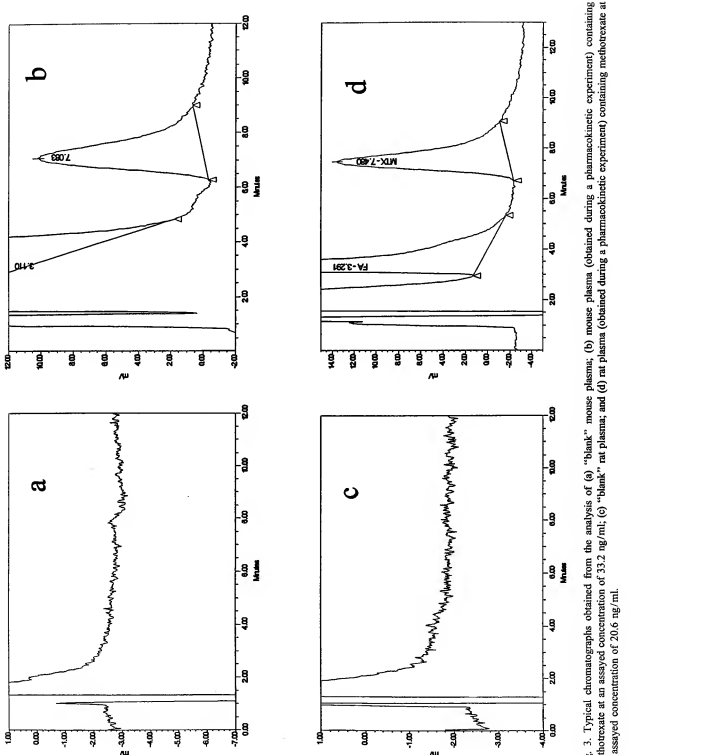


Fig. 3. Typical chromatographs obtained from the analysis of (a) "blank" mouse plasma; (b) mouse plasma (obtained during a pharmacokinetic experiment) containing methotrexate at an assayed concentration of 33.2 ng/mL; (c) "blank" rat plasma; and (d) rat plasma (obtained during a pharmacokinetic experiment) containing methotrexate at an assayed concentration of 20.6 ng/mL.

Table 1
Accuracy and precision for MTX in rat plasma

| QC (ng/ml) | Mean assay response | SD | RSD (%) | Recovery (%) |
|---|---|---|---|---|
| *(A) Total MTX in presence of AMF* | | | | |
| Intra-day variability | | | | |
| 500 | 508.5 | 8.7 | 1.7 | 101.7 |
| 250 | 267.2 | 6.4 | 2.4 | 106.9 |
| 100 | 102.9 | 5.4 | 5.2 | 102.9 |
| 25 | 26.7 | 2.1 | 7.9 | 106.9 |
| Inter-day variability | | | | |
| 500 | 515.5 | 9.4 | 1.8 | 103.1 |
| 250 | 267.7 | 19.4 | 7.2 | 107.1 |
| 100 | 98.3 | 2.7 | 2.7 | 98.3 |
| 25 | 25.3 | 1.4 | 5.7 | 101.1 |
| *(B) Total MTX in absence of AMF* | | | | |
| Intra-day variability | | | | |
| 500 | 508.5 | 8.7 | 1.7 | 101.7 |
| 100 | 102.9 | 5.4 | 5.2 | 102.9 |
| 25 | 26.7 | 2.1 | 7.9 | 106.9 |
| Inter-day variability | | | | |
| 500 | 515.5 | 9.4 | 1.8 | 103.1 |
| 100 | 98.3 | 2.7 | 2.7 | 98.3 |
| 25 | 25.3 | 1.4 | 5.7 | 101.1 |

Table 2
Accuracy and precision for MTX in mouse plasma

| QC (ng/ml) | Mean assay response | SD | RSD (%) | Recovery (%) |
|---|---|---|---|---|
| *(A) Total MTX in presence of AMF* | | | | |
| Intra-day variability | | | | |
| 250 | 251.2 | 18.5 | 7.4 | 100.5 |
| 50 | 50.9 | 4.5 | 8.9 | 101.9 |
| 25 | 24.5 | 1.3 | 5.4 | 98.0 |
| Inter-day variability | | | | |
| 250 | 270.9 | 13.9 | 5.2 | 108.4 |
| 50 | 54.9 | 0.5 | 0.9 | 109.9 |
| 25 | 26.3 | 1.6 | 6.0 | 105.2 |
| *(B) Total MTX in absence of AMF* | | | | |
| Intra-day variability | | | | |
| 250 | 262.3 | 13.9 | 5.3 | 104.9 |
| 100 | 100.4 | 2.5 | 2.5 | 100.4 |
| 25 | 26.0 | 1.9 | 7.5 | 104.0 |
| Inter-day variability | | | | |
| 250 | 259.0 | 10.5 | 4.1 | 103.6 |
| 100 | 97.9 | 2.7 | 2.8 | 97.9 |
| 25 | 26.4 | 1.2 | 4.7 | 105.8 |

Table 3
Accuracy and precision for free MTX

| QC (ng/ml) | Mean assay response | SD | RSD (%) | Recovery (%) |
|---|---|---|---|---|
| *(A) In rat plasma* | | | | |
| Intra-day variability | | | | |
| 500 | 549.4 | 5.2 | 0.9 | 109.9 |
| 100 | 107.8 | 2.5 | 2.3 | 107.8 |
| 50 | 54.9 | 1.1 | 2.0 | 109.7 |
| Inter-day variability | | | | |
| 500 | 548.5 | 28.6 | 5.2 | 109.7 |
| 100 | 109.8 | 1.3 | 1.2 | 109.8 |
| 50 | 52.9 | 2.3 | 4.3 | 105.9 |
| *(B) In mouse plasma* | | | | |
| Intra-day variability | | | | |
| 250 | 272.6 | 12.7 | 4.7 | 109.0 |
| 100 | 101.1 | 4.7 | 4.6 | 101.1 |
| 25 | 26.4 | 0.9 | 3.3 | 105.5 |
| Inter-day variability | | | | |
| 250 | 260.9 | 26.5 | 10.2 | 104.3 |
| 100 | 97.9 | 7.4 | 7.6 | 97.9 |
| 25 | 26.2 | 0.8 | 2.9 | 104.6 |

### 3.4. Pharmacokinetics in rats

The concentration–time profile of MTX after i.a. administration of 2.0 μmol/kg (909 μg/kg) is shown in Fig. 5. Fitting the data to a two compartment model resulted in clearance value of 1.0 l/kg/h, volume of distribution in the central compartment of 0.41 l/kg and half life of 28 min. These values compare well with those reported by Slordal et al. in male Wistar rats at a dose of 1.0 mg/kg with clearance of 1.16±0.14 l/kg/h, central volume of distribution of 0.26±0.04 l/kg and half life of 25.6±3.0 min [29]. The disposition of MTX following continuous i.a. infusion of AMF and bolus MTX

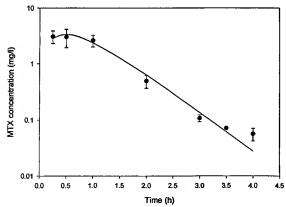


Fig. 4. MTX concentration time profile in mice following an i.p. bolus of 10 mg/kg; $n=3$. Data were fitted to a two-compartment mammillary model using the Scientist program.

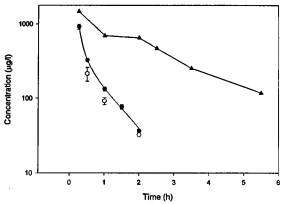


Fig. 5. MTX concentration time profile in rat with continuous i.a. infusion of anti-MTX Fab fragment over 6 h and i.a. bolus of 2.0 μmol/kg MTX. The fit concentration time profile is shown as the smooth line, while observed concentrations are represented as closed circles (total MTX concentration, following MTX administered alone), open circles (free MTX concentration, following MTX administered alone), and closed triangles (total MTX concentration, following AMF and MTX administration).

is shown in Fig. 5. Non-compartmental fitting of the data gave a clearance value of 0.32 l/kg/h. The low clearance of MTX in the presence of AMF is due to the slow elimination of MTX bound to the antibody. With MTX administration alone, the free MTX fraction at 2 h was 88% while in presence of the antibody free MTX fraction was less than 1%. This is in agreement with our hypothesis that circulating anti-MTX antibody will reduce the free fraction of MTX in the systemic circulation.

## 4. Conclusion

In this paper, we have presented a sensitive and validated HPLC method to analyze total and free MTX in rat and mouse plasma in the presence and absence of anti-MTX antibody fragments. This is the only HPLC assay, to our knowledge, that is capable of measuring both free and total MTX in rat and mouse plasma. The assay will enable the testing our pharmacokinetic hypotheses regarding the influence of anti-MTX Fab fragments on the disposition of MTX in these species.

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